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(71) Applicants and
(72) Inventors: **YANG, Dajun** [CN/US]; 10330 Royal Woods Court, Gaithersburg, MD 20886 (US). **ZHU, Xiao, F.** [CN/CN]; Cancer Center, SUMS, Guangzhou (CN). **WANG, Jingson** [CN/CN]; First Affil Hospital, Guangzhou (CN). **ZENG, Yixing** [CN/CN]; Cancer Center, SUMS, Guangzhou (CN).

(74) Agents: **TESKIN, Robin, L.** et al.; Pillsbury Winthrop LLP, 1600 Tysons Boulevard, McLean, VA 22102 (US).

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(54) Title: HOUTTUYNINUM COMPOSITIONS AND METHODS FOR INHIBITING THE ACTIVITY OF ERBB-2 BASED THEREON

(57) Abstract: The present invention relates to methods and compositions for inhibiting the activity of erbB-2. The described methods and compositions are particularly useful for inhibiting cellular proliferative disorders, such as cancer, characterized by over-activity and/or inappropriate activity of erbB-2. More particularly, method for the treatment of a cellular proliferative disorder characterized by over-activity or inappropriate activity of erbB-2 is provided including the step of administering to a patient in need of such treatment a therapeutically effective amount of soluble extract of houttuynum, or a compound selected from the group consisting of houttuyninum, Houttuymia cordata, neo-houttuyninum (decanoyl acetaldehyde), analogs thereof, pharmaceutically acceptable salts thereof, and/or prodrugs thereof.

HOUTTUYNINUM COMPOSITIONS AND METHODS FOR INHIBITING THE ACTIVITY OF ERBB-2 BASED THEREON

Field of the Invention

[0001] The present invention relates to methods and compositions for inhibiting the activity of erbB-2. The described methods and compositions are particularly useful for inhibiting cellular proliferative disorders, such as cancer, characterized by over-activity and/or inappropriate activity of erbB-2.

Background of the Invention

[0002] For mammalian cells to survive, they must be able to respond rapidly to changes in their environment. Furthermore, for cells to reproduce and carry out other cooperative functions, they must be able to communicate efficiently with each other. Cells most frequently adapt to their environment and communicate with one another by means of chemical signals. An important feature of these signaling mechanisms is that in almost all cases a cell is able to detect a chemical signal without it being necessary for the chemical messenger itself to enter the cell. This permits the cell to maintain the homeostasis of its internal environment, thereby permitting the cell to respond to its external environment without being adversely affected by it.

[0003] These sensing functions are carried out by a variety of receptors, which are dispersed on the outer surface of the cell and function as "molecular antennae." These receptors detect an incoming messenger and activate a signal pathway that ultimately regulates a cellular process such as secretion, contraction, metabolism or growth.

[0004] In the cellular plasma membrane, transduction mechanisms translate external signals into internal signals, which are then carried throughout the interior of the cell by chemicals known as "second messengers." In molecular terms, the process depends on a series of proteins within the cellular plasma membrane, each of which transmits information by inducing a conformational change in the protein next in line. At some point, the information is assigned to small molecules or even to ions within the cell's cytoplasm, which serve as the above-mentioned second messengers. The diffusion of the second messengers enables a signal to propagate rapidly throughout the cell.

[0005] Abnormal cell signaling has been associated with cellular proliferative disorders, such as cancer. Cell signaling plays a crucial role in cell growth, proliferation and differentiation. Thus, when normal cell signaling pathways are altered, uncontrolled cell growth, proliferation and/or differentiation can take place, leading to the formation and propagation of cancer.

[0006] Cancer is the leading cause of death, second only to heart disease in both men and women. Breast cancer is the most common tumor in women, representing 32% of all new cancer cases and causing 18% of cancer-related deaths of women in the United States. In the fight against cancer, numerous techniques have been developed and are the subject of current research to understand the nature and cause of the disease, and to provide techniques for the control or cure thereof. One promising avenue for the development of cancer treatments is based on blocking abnormal cell signaling pathways. Particular efforts are directed to the elucidation and regulation of the activity of receptor and transmembrane proteins.

[0007] For instance, the human epidermal growth factor (EGF) is a six kilodalton (kDa), 53 amino acid, single-chain polypeptide

which exerts its biological effect by binding to a specific 170 kDa cell membrane receptor (EGF-R). The human EGF-R consists of an extracellular domain with a high cysteine content and N-linked glycosylation, a single transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity.

[0008] Many types of cancer display enhanced EGF-R expression on their cell surface membranes. Enhanced expression of the EGF-R can increase signaling via receptor-mediator pathways that lead to pleiotropic biological effects including excessive proliferation and metastasis. Examples include prostate cancer, breast cancer, lung cancer, head and neck cancer, bladder cancer, melanoma, and brain tumors.

[0009] In breast cancer, expression of the EGF-R is a significant and independent indicator for recurrence and poor relapse-free survival. The epidermal growth factor receptor (EGF-R) of cancer cells therefore represents a potential target for biotherapy.

[0010] EGF-R and its physiologic ligands, epidermal growth factor (EGF) and transforming growth factor alpha (TGF alpha), play a prominent role in the growth regulation of many normal and malignant cell types. One role the EGF receptor system may play in the oncogenic growth of cells is through autocrine-stimulated growth. Cells that express EGF-R and secrete EGF and/or TGF alpha can stimulate their own growth, thereby creating a cancerous condition. An autocrine growth stimulatory pathway analogous with that proposed for epidermal growth factor receptor and its ligands may also be employed by a growing list of oncogene encoded transmembrane proteins that have a structure reminiscent of that of the growth factor receptors.

[0011] One such example is the HER-2/neu or c-erbB-2 oncogene, which belongs to the erbB-like oncogene group, and is related to, but distinct from EGF-R. The erbB-2 gene encodes a 185 kD transmembrane glycoprotein that has partial homology with other members of the EGFR family. The expressed protein has been suggested to be a growth factor receptor due to its structural homology with EGFR. However, known EGFR ligands, such as EGF or TGFα do not bind to p^{185}-erbB-2.

[0012] The erbB-2 oncogene has been demonstrated to be implicated in a number of human adenocarcinomas leading to elevated levels of expression of the p^{185} protein product. For example, the erbB-2 oncogene has been found to be amplified in breast, ovarian, gastric and even lung adenocarcinomas. Furthermore, the amplification of the c-erbB-2 oncogene has been found in many cases to be a significant, if not the most significant, predictor of both overall survival time and time to relapse in patients suffering from such forms of cancer. Carcinoma of the breast and ovary account for approximately one-third of all cancers occurring in women and together are responsible for approximately one-fourth of cancer-related deaths in females.

[0013] Significantly, the c-erbB-2 oncogene has been found to be amplified in 25 to 30% of human primary breast cancers and it has been associated with a high risk of relapse and death. In breast cancers with erbB-2 overexpression abnormal cell proliferation is believed to be caused by extremely high tyrosine kinase activity and the resulting high level of signal transduction. Overexpression of erbB-2 has also been found to be associated with increased resistance to chemotherapy or patients with elevated levels of erbB-2 respond poorly to many drugs. It is believed that decreasing the levels of erbB-2 will allow

chemotherapeutic drugs to be more effective. Therefore, therapies targeted at erbB-2 have the great therapeutic potential for the treatment of breast cancers.

[0014] In view of the above, the development of new and potent anti-breast cancer drugs and the design of treatment protocols directed at the inhibition of erbB-2 activity is an exceptional focal point for research in the modern therapy of breast cancer. Drug targeting is a particularly attractive approach for killing malignant cells, when leaving normal tissue unharmed is achieved.

[0015] As such, erbB-2 is a clinically proven therapeutic target for breast cancer. Indeed, the recently completed clinical trial of anti-Her2 Herceptin provide evidence that systemic administration of Herceptin, alone and in combination with cytotoxic chemotherapy in patients with erbB-2 overexpressing primary tumors, can increase the time to recurrence and overall response rates in metastatic breast cancer. Herceptin is recognized as the first in what promises to-be a wave of therapies attacking cancer at its genetic roots.

[0016] In breast cancers with erbB-2 overexpression, abnormal cell proliferation is caused by the extremely high tyrosine kinase activity and resulting high level of signal transduction. Drugs blocking this extremely high erbB-2 tyrosine kinase activity could have the potential to shut down signaling pathways mediated by erbB-2. Thus, erbB-2 kinase inhibitor compounds that are capable of entering the cell, blocking tyrosine kinase activity and shutting down the signal transduction pathway mediated by erbB-2 may be used as potential therapeutic agents for the treatment of breast cancer. Furthermore, it has been shown that tyrosine kinase inhibitors synergize with antibodies to EGF-R to inhibit the growth of aquamous cell carcinoma *in*

vivo. Thus, a specific erbB-2 kinase inhibitor may also have synergistic effects with Herceptin in the treatment of breast cancer.

[0017] Nonetheless, certain limitations are associated with large molecule strategies, including poor delivery, poor *in vivo* stability, possible immune response and high cost. Accordingly, it is highly desirable to provide therapies based on small molecules targeted at interfering with erbB receptor-mediated signal transduction pathways (including erbB-2, erbB-3 and erbB-4). Compared to therapies based on large drug molecules, such as therapeutic antibodies, small molecule drug therapies have a number of advantages, including good oral availability and low cost. A number of criteria should thus be considered in the development of erbB-2 kinase inhibitor compounds, including good potency, selectivity, cell permeability, bioavailability, appropriate pharmacokinetics and non-toxicity.

[0018] The development of small molecule kinase inhibitors of the EGF-R family of receptor tyrosine kinases has been so far focused on EGF-R itself. Very potent and selective EGF-R small molecule kinase inhibitors have been reported and some EGFR small molecule kinase inhibitors have advanced to clinical trials for the treatment of certain cancer forms. To date, very few kinase inhibitors selective for erbB-2 have been reported. Therefore, it would be greatly beneficial if new therapies could be designed using compounds that are active as erbB-2 kinase inhibitors. In particular, therapies using compounds having improved selectivity, solubility and stability would be desirable.

Summary of the Invention

[0019] It is an object of the invention to provide novel therapies and compositions capable of inhibiting the erbB-2 kinase signaling pathway.

[0020] It is a more specific object of the invention to provide novel therapies that result in the inhibition of cell proliferation and/or differentiation and/or the promotion of cell apoptosis comprising the administration of a compound that inhibits erbB-2 kinase related cell growth signaling.

[0021] It is yet another object of the invention to provide novel therapies that result in the inhibition of cell proliferation and/or differentiation and/or promotion of cell apoptosis by the administration of soluble extracts of houttuynum, or a compound selected from the group consisting of houttuyninum, Houttuymia cordata, neo-houttuyninum (decanoyl acetaldehyde), analogs thereof, pharmaceutically acceptable salts thereof, and/or prodrugs thereof.

[0022] In a preferred embodiment, such therapies will comprise treatment of cancer and other neoplastic conditions.

Brief Description of the Drawings

[0023] Figure 1 shows the structure of houttuyninum.

[0024] Figure 2 shows the inhibition of erbB-2 phosphorylation in BT-474 and MDA-453 cells by houttuyninum compound.

[0025] Figure 3 shows the inhibition of MAPK activation in BT-474 and MDA-453 cells by houttuyninum compound.

[0026] Figure 4 shows the inhibition of AKT activation in BT-474 and MDA-453 cells by houttuyninum compound.

[0027] Figure 5 shows the inhibition of HER2 phosphorylation in MDA-453 cells by houttuyninum compound.

[0028] Figure 6 shows the inhibition of EGFR phosphorylation in A431 and MDA-468 cells by houttuyninum compound.

[0029] Figure 7 shows the inhibition of HER2 activation MDA-453 cells by houttuyninum compound.

[0030] Figure 8 shows the inhibition of MAPK activation MDA-453 cells by houttuyninum compound.

[0031] Figure 9 shows the inhibition of AKT activation MDA-453 cells by houtttuyninum compound.

[0032] Figure 10 shows the inhibition of HER2 activation in BT-474 cells by houttuyninum compound.

[0033] Figure 11 shows the inhibition of MAPK activation BT-474 cells by houttuyninum compound.

[0034] Figure 12 shows the inhibition of AKT activation BT-474 cells by houtttuyninum compound.

[0035] Figure 13 shows the inhibition of erbB-2 phosphorylation in AKT activation BT-474 cells by the soluble extract of houtttuynum.

[0036] Figure 14. Effects of the soluble extract houttuynum (YX1, 2mg/ml in original stock) on phosphorylation of erbB-2 in MDA-453 or EGFR in MDA-468 cells.

[0037] Figure 15. Effects of the soluble extract houttuynum (YX1 2 mg/ml in original stock) on protein expression of erbB-2 in MDA-453 or EGF-R in MDA-468 cells.

[0038] Figure 16. Enhanced cytotoxicity of Doxrubicin in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).

[0039] Figure 17. Enhanced cytotoxicity of Cisplatin in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).

[0040] Figure 18. Enhanced cytotoxicity of VP-16 in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).

[0041] Figure 19. Effect of the soluble extract houttuynum (YX1, 2 mg/ml in original stock) or houttuyninum compound (YX2) on *in vivo* tumor proliferation of erbB-2 overexpressing human cancer cell line N87.

Detailed Description of the Invention

[0042] As described above, the overexpression of erbB-2, which encodes tyrosine kinase receptor pl85neu, plays a key role in tumor generation or progression such as breast cancer, ovarian cancer, *etc.* To explore the potential of therapy targeting erbB-2 kinase, it was unexpectedly discovered that houttuynum, Houttuymia cordata, and neo-houttuyninum (decanoyl acetaldehyde) selectively suppressed autophosphorylation of erbB-2 tyrosine kinase in breast cancer cell lines MDA-453 and BT-474, and thus inhibited the activity of erbB-2.

[0043] Thus, a first aspect of the invention provides therapies using compounds capable of inhibiting erbB-2 kinase activity. Such compounds according to the invention include soluble

extract of houttuynum, or compound houttuyninum, Houttuymia cordata, neo-houttuyninum (decanoyl acetaldehyde), analogs thereof, pharmaceutically acceptable salts thereof, and/or prodrugs thereof.

[0044] Houttuynum is an antibacterial agent from Chinese herbal medicine that has been shown to have non- or low toxicity to humans. Houttuynia cordata is an antimicrobial agent from Chinese herbal medicine that has been used to treat various infections such as bronchitis, pneumonia, whooping cough, and urinary infections.

[0045] In a preferred embodiment, a therapy according to the invention can involve a method for the treatment of a cellular proliferative disorder characterized by over-activity or inappropriate activity of erbB-2 comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound selected from the group consisting of houttuyninum, Houttuymia cordata, neo-houttuyninum (decanoyl acetaldehyde), analogs thereof, pharmaceutically acceptable salts thereof, and/or prodrugs thereof.

[0046] As used herein, "cellular proliferative disorders" refer to disorders wherein unwanted cellular proliferation of one or more subset(s) of cells in a multicellular organism occurs, resulting in harm (*e,g.*, discomfort or decreased life expectancy) to the multicellular organism and can include, for example, cancers, blood vessel proliferative disorders, and fibrotic disorders. A particular disorder is considered to be "driven" or caused by a particular receptor tyrosine kinase if the disorder is characterized by over-activity, or inappropriate activity, of the kinase. The term "inappropriate activity" refers to either: 1) erbB-2 expression in cells which normally do not express such receptors; 2) increased erbB-2 expression leading to unwanted

cell proliferation such as cancer; 3) increased erbB-2 activity leading to unwanted cell proliferation, such as cancer; and/or over-activity of erbB-2. The term "over-activity" of erbB-2 refers to either an amplification of the gene encoding the specific receptors or the production of a level of erbB-2 activity which can be correlated with a cell proliferative disorder (*i.e.*, as the level of erbB-2 increases, the severity of one or more of the symptoms of the cell proliferative disorder increases).

[0047] Another aspect of the invention provides a class of erbB-2 inhibitor compositions comprising, in admixture with a pharmaceutically acceptable carrier, a therapeutically effective amount of a compound that is capable of inhibiting erbB-2 activity. Again, such compounds according to the invention include soluble extract of houttuynum, houttuyninum, Houttuynia cordata, neo-houttuyninum (decanoyl acetaldehyde), analogs thereof, pharmaceutically acceptable salts thereof, and/or prodrugs thereof. The term "therapeutically effective" generally refers to either the inhibition, to some extent, of growth of cells causing or contributing to a cell proliferative disorder; or the inhibition, to some extent, of the activity of erbB-2.

[0048] Generally, inhibition of erbB-2 kinase autophosphorylation by the compounds of the invention has been found to be selective and dose-dependent. Further, activation of molecules downstream of the erbB-2 receptor pathway, *e.g.*, MAPK and AKT, has also been found to be inhibited by such compounds.

[0049] For instance, as shown in the examples below, houttuynum was unexpectedly found to inhibit erbB-2 phosphorylation. ErbB-2 autophosphorylation was shown to be inhibited by houttuynum in different breast cancer cell lines, such as MDA-453 (MDA-453), and BT-474 (BT-474). At

concentrations of at least 2.5 μg/ml, houttuynum obviously inhibited erbB-2 kinase. Further, the effect of houttuynum on EGF-R tyrosine kinase activity (in cells such as MDA-468 or A431 which overexpressing EGF-R) was detected in order to elucidate the selectivity of houttuynum directed against erbB-2. The results demonstrate that houttuynum does not have an inhibitory effect on EGF-R tyrosine kinase under the same dosages and conditions, *i.e.*, dosages under 20 μg/ml.

[0050] Growth inhibition of MDA-453 cells, which overexpress erbB-2, and MDA-468 cells, which overexpress EGF-R, by houttuynum was also investigated using cell proliferation assay (MTT assay). The results show that houttuynum inhibits growth of MDA-453 cells more than that of MDA-468. Thus, it was unexpectedly discovered that houttuynum inhibits tyrosine kinase activity of erbB-2 receptor and the growth of breast cancer cell lines overexpressing erbB-2 receptor preferentially. Further, the study of irreversibility or reversibility shows that inhibition of erbB-2 phosphorylation by houttuynum is reversible and that the expression of erbB-2 receptor protein in breast cancer cell lines MDA-453 and BT-474 is unaffected following treatment with houttuynum. This effect is different from other tyrosine kinase inhibitors such as AAG-17 or gadelamycin.

[0051] Thus, the compounds according to the invention can be used to treat cellular proliferative disorders wherein inhibition of erbB-2 kinase signaling is therapeutically beneficial. This will include disorders that involve abnormal cell growth and/or differentiation, such as cancers and other neoplastic conditions. Also, the subject compounds may be used to treat other conditions involving abnormal cell proliferation and/or differentiation, such as dermatological conditions and disorders. Further, the subject compounds may be useful in treating

inflammatory conditions such as arthritis, psoriasis, autoimmune disorders such as myasthenia gravis, lupus, multiple sclerosis, and others, and conditions involving abnormal platelet aggregation. The preferred indication is cancer, especially cancers involving over-expression of erbB-2, EGF, and/or the PDGF receptor, cancers that express mutant *ras*, or cancers that comprise a Bcr/Abl translocation. Examples of cancers that may be treated according to the invention include breast, colon, pancreatic, prostate, head and neck, gastric, renal, ovary, brain and lung cancers.

[0052] The subject therapies can comprise the administration of soluble extract, and/or at least one compound according to the invention in a therapeutically effective amount, *e.g.*, an amount sufficient to inhibit tumor cell proliferation and/or differentiation and/or promotion of apoptosis. The soluble extract or compound may be administered by any pharmaceutically acceptable means, by either systemic or local administration. Suitable modes of administration include oral, dermal , *e.g.*, via transdermal patch, inhalation, via infusion, intranasal, rectal, vaginal, topical parenteral (*e.g.*, via intraperitoneal, intravenous, intramuscular, subcutaneous, injection).

[0053] Typically, oral administration or administration via injection is preferred. The subject compounds may be administered in a single dosage or chronically dependent upon the particular disease, condition of patient, toxicity of compound, and whether this compound is being utilized alone or in combination with other therapies. Chronic or repeated administration will likely be preferred based on other chemotherapies.

[0054] In a preferred embodiment, the subject compounds can be administered as erbB-2 inhibitor compositions comprising, in

admixture with a pharmaceutically acceptable carrier, a therapeutically effective amount of a compound of the invention. Examples of such compositions include injectable solutions, tablets, milk, or suspensions, creams, oil-in-water and water-in-oil emulsions, microcapsules and microvesicles. These compositions can comprise conventional pharmaceutical excipients and carriers typically used in drug formulations, *e.g.*, water, saline solutions, such as phosphate buffered saline, buffers, and surfactants.

[0055] The subject compounds may be free or entrapped in microcapsules, in colloidal drug delivery systems such as liposomes, microemulsions, and macroemulsions. Suitable materials and methods for preparing pharmaceutical compositions are disclosed in *Remington's Pharmaceutical Chemistry*, 16th Edition, (1980). Also, solid compositions containing the subject compounds, such as tablets, and capsule formulations, may be prepared. Suitable examples thereof include semipermeable materials of solid hydrophobic polymers containing the subject compound which may be in the form of shaped articles, *e.g.*, films or microcapsules, as well as various other polymers and copolymers known in the art.

[0056] Further, the compounds described herein can be used alone, in combination with other pharmaceutically active agents able to inhibit protein kinase activity (*e.g.*, anti-sense nucleic acid and ribozymes targeted to nucleic acid encoding a receptor tyrosine kinase, and antibodies able to modulate tyrosine kinase activity, such as anti-erbB-2 antibodies which may work by modulating erbB-2, and in combination with other types of treatment for cell proliferative disorders, such as chemotherapy.

[0057] For instance, several chemotherapeutic agents are known in art for treating breast cancer, including doxorubicin,

cyclophosphamide, methotrexate, 5-fluorouracil, mitomycin C, mitoxantrone, taxol, and epirubicin. Numerous chemotherapeutic agents are also known in the art for treating different types of leukemia, including the treatment of AML using daunorubicin, cytarabine (Ara-C), doxorubicin, amsacrine, mitoxantrone, etoposide (VP-16), thioguanine, mercaptopurine, and azacytidine; the treatment of ALL using vincristine, prednisone, doxorubicin and asparginase; the treatment of CML using busulfan and hydroxyurea; and the treatment of CLL using chlorambucil and cyclophosphamide. Additional treatments include use of alpha-interferon, bone marrow transplantation and transplantation of peripheral blood or umbilical cord blood stem cells.

[0058] The dosage effective amount of compounds according to the invention will vary depending upon factors including the particular compound, toxicity, and inhibitory activity, the condition treated, and whether the compound is administered alone or with other therapies. Typically a dosage effective amount can be at least about 0.25 μg/ml, preferably from about 2.5 μg/ml to about 400 μg/ml, and more preferably about 5 μg/ml to about 50 μg/ml. When used at such concentrations, the compounds of the invention can preferentially and selectively inhibit erbB-2 activity.

[0059] The subjects treated will typically comprise mammals, and most preferably will be human subjects, *e.g.*, human cancer subjects.

[0060] The invention will now be described in more detail with reference to the use of houttuynum to inhibit the activity of erbB-2 in the following examples.

EXAMPLES

Cell culture and reagents

[0061] The human breast cancer cell lines MDA-453 (also known as MDA-MB-453ml), BT-474 (also known as BT-474ml), MDA-468 and A431 were obtained from American Type culture collection (Rockville, MD). BT-474 was grown in DMEM medium (Life Technologies, Inc. Grand Island, NY) supplemented with 10% fetal bovine serum). MDA-453, MDA-468 and A431 were cultivated in RMPI-1640 medium (Life Technologies, Inc.). Cells were grown in an incubator at 37 °C under 5% C02 in air. ErbB-2 antibody was obtained from Calbiochem Co. Anti-tyrosine antibody (PY99) was purchased from Santa Cruz. Anti-Akt, phospho-Akt, MAPK and phospho-MAPK antibody was purchased from BioLab co. Houttuynum was synthesized in house.

Western blot analysis

[0062] Cells were seeded at a density of 2 x 10^5 cells/well in 12-well plate. The next day, the regulatory medium was aspirated and replaced with serum-free medium. Drug stock was added to indicated concentrations. After treatment with houttuynum for 1 hour, cells were washed twice with PBS, and lysed in 100 ul of lysis buffer (20 mM Na2PO4(pH 7.4), 150mM NaCl, 1% Triton X-l00, 1% aprotinin, 1mM phenymethysulfonyl fluoride, 10 mg/mL leupeptin, 100 mM NaF, and 2 mM Na3VO4). Lysates were centrifuged at 12000 rpm for 10 min. The supernatant was collected. The protein content was determined using the Bio-Rad protein assay (Bio-Rad laboratories, Hercules CA). A total of 40 μg of protein was resolved by SDS-PAGE using 4-20% gel and transferred electrophoretically to nitrocellulose membrane (Amersham Co.). The membranes were blotted for 30 min at 25

°C with nonfat dry milk (5%) in TBST (20mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1% Tween), and then incubated with primary antibodies for 2 hours at 25 °C. The membranes were washed with TBST and incubated horseradish perioxidase-labeled anti-mouse or rabbit secondary antibody for 1 hour at 25 °C. Being washed with TBST, the bound antibody complex was detected using an ECL chemiluminescence reagent and XAR film (Kodak) as described by the manufacturer (Amersham).

Irreversibility/reversibility of inhibition of erbB-2 tyrosine kinase

[0063] MDA-453 cells were plated at a density of 5 x 10^5 cells per well in 12-well plate. The next day, then regular medium was changed into serum-free medium. Drug stock was added. Following treatment with l0ug/ml of houttuynum for 30 min, cells were washed twice with serum-free medium. 1 ml of serum-free medium was added and incubated in an incubator at 37 °C under 5% C02 in air for different periods of time. Cells were lysed and western blot assay was conducted.

MTT assay

[0064] Cells were plated at a density of 2000 cells/well in 96-well plate. The next day, the medium was replaced with serum-free medium. The stock of houttuynum was diluted with medium, then added to the wells for the desired final assay concentrations. After 7 days of exposure to houttuynum, 10 uL of 5 mg/mL MTT was added to each well and incubated for four more hours, and the liquid in the wells was evaporated. 100 μL of DMSO was added to each well to dissolve the formazam . The absorbance was detected in the microplate reader 450 model with 565nm wavelength. Growth inhibition was expressed as a percentage of absorbance detected in the control wells that were

treated with 0.1% DMSO alone. DMSO controls were not different from cells in the regular growth medium.

Results

[0065] To examine whether houttuynum can inhibit erbB-2 tyrosine kinase activity, MDA-453, BT-474, SKBr3, MDA-361 human breast cancer cells that overexpress $p185^{neu}$ were used to test the effect of houttuynum on tyrosine phosphorylation of erbB-2. To monitor the effects of kinase inhibition, the degree of erbB-2 autophosphorylation was conducted. This is because autophosphorylation of effector-specific tyrosine residues increases, the velocity of the kinase reaction and autophosphorylation increases the affinity of erbB-2 for its substrates (such as phospholipase-C, Grb-2, Shc, etc.), allowing these substrates to bind to the activated receptor. Cells were treated with varying concentrations of houttuynum at 37 °C for 30 min, then analyzed for the protein level of $p185^{neu}$ and phosphorylation of erbB-2. ErbB-2 protein phosphorylation levels were detected with anti-phospho-erbB-2 antibody. Houttuynum inhibited tyrosine phosphorylation of $p185^{neu}$ significantly under the concentrations of 5,10 μg/ml in MDA-453 and BT-474 (Figs. 1, 6, and 9). To confirm inhibition of phosphorylation by houttuynum, but not expression of $p185^{neu}$ protein, the same filter was stripped, and was blocked with 5% fat-free milk. With anti-erbB-2 antibody as the primary antibody, erbB-2 protein level was detected using Western blot analysis. The results showed that erbB-2 protein expression had no change following houttuynum treatment.

[0066] As shown above, houttuynum can inhibit erbB-2 kinase. To explore whether it can also inhibit EGF-R tyrosine kinase activity, the effect of houttuynum on the tyrosine kinase activity

of EGF-R was tested. The inhibitory effect on EGF-R tyrosine kinase activity was found to be weak under 20 ug/ml of concentration of houttuynum. When the concentration escalated to 100 ug/ml, houttuynum had a significant inhibitory effect on EGF-R tyrosine kinase. However, under 20 ug/mL of concentration, houttuynum inhibited tyrosine kinase of erbB-2 significantly. Our results suggest that houttuynum inhibits erbB-2 tyrosine kinase preferentially (Fig. 5).

[0067] To test the effect of houttuynum on MAPK activation, a downstream target of the erbB-2 signal pathway, cells were treated with varying concentrations of houttuynum for one hour, MAPK activation was detected with anti-phospho-MAPK antibody and anti-phospho-MAPK in MDA-453ml and BT-474 breast cancer cell lines. Phospho-MAPK is the active type of MAPK. The results showed that phospho-MAPK decreased significantly after treatment of cells with houttuynum, while total MAPK levels were unaffected (Figs. 2, 7, and 10).

[0068] To test the effect of houttuynum on AKT activation, a downstream target of the erbB-2 signal pathway, cells were treated with varying concentrations of houttuynum for one hour, AKT activation was detected with anti-phospho-AKT antibody and anti-AKT antibody in MDA-453 and BT-474 breast cancer cell lines. Phospho-AKT is the active type of AKT. The results showed that phospho-AKT decreased significantly after treatment of cells with houttuynum, while total Akt levels were unaffected (Figs. 3, 8, and 11).

[0069] The reversibility of the inhibition of erbB-2 by houttuynum was also investigated. MDA-453 cells were treated with houttuynum for 30 mm, the wells were washed with serum-free medium, then incubated for one hour, three hours, eight hours, and 24 hours separately. The phosphorylation of tyrosine

kinase of erbB-2 was detected. The results showed that the phosphorylation of erbB-2 kinase could not recover following treatment of houttuynum for 30 minutes (Fig. 4).

[0070] The ability of houttuynum to preferentially inhibit cell growth in cells that overexpress erbB-2 was also investigated. MDA-453 cells, which overexpress erbB-2, and MDA-468 cells, which overexpress EGF-R, were treated with houttuynum, and cell growth was then monitored. The results indicated that houttuynum inhibited the growth of MDA-453 cells to a greater extent than it inhibited the growth of MDA-468 cells, thus demonstrating the preferential inhibition of cell growth in cell lines that overexpress erbB-2.

[0071] Also within the scope of the invention is the use of the compounds described herein to determine whether a cellular proliferative disorder is driven, to some extent, by erbB-2. A diagnostic assay to determine whether a particular disorder is driven by erbB-2 can be carried out using the following steps: 1) culturing test cells or tissues; 2) administering a compound according to the invention which can selectively inhibit erbB-2 activity; and 3) measuring the degree of growth inhibition of the test cells. These steps can be carried out using standard techniques in light of the present disclosure. For example, standard techniques can be used to isolate cells or tissues and culturing in vitro or in vivo.

[0072] While the invention has been described in terms of preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions and changes may be made without departing from the spirit thereof. Accordingly Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims, including equivalents thereof.

WHAT IS CLAIMED IS:

1. A method for treating a cellular proliferative disorder characterized by the over-activity and/or inappropriate activity of erbB-2 comprising the step of administering to a patient in need of such treatment a therapeutically effective amount of at least one compound capable of selectively inhibiting the activity of erbB-2, selected from the group consisting of soluble extract of houttuynum, or compound houttuyninum, Houttuynia cordata, neo-houttuyninum, analogs thereof, pharmaceutically acceptable salts thereof, and/or prodrugs thereof.

2. The method of claim 1 wherein the compound is administered to the patient in an amount of at least about 0.25 μg/ml, per kilogram of patient weight.

3. The method of claim 1, wherein said selective inhibiting antagonizes erbB-2 cell growth signaling.

4. The method of claim 1, wherein said method results in the inhibition of cell proliferation and/or differentiation.

5. The method of claim 1, which results in the induction of apoptosis.

6. The method of claim 1, which said cellular proliferative disorder is cancer.

7. The method of Claim 6, wherein said cancer is characterized by cells that over-express the erbB-2 and/or the EGF receptor, cells that express a mutant *ras*, or cells that have abnormal expression of c-AMP, or cells that comprise a Bcr/Abl transfection.

8. The method of claim 6, wherein the cancer is selected from the group consisting of breast cancer, prostate cancer, lymphoma, skin cancer, pancreatic cancer, colon cancer, melanoma, ovarian cancer, brain cancer, liver cancer, bladder

cancer, non-small lung cancer, gastric, head and neck, cervical carcinoma, leukemia and neuroblastoma/glioblastoma.

9. The method of claim 1, wherein said compound is administered by a method selected from the group consisting of oral, intranasal, intraperitoneal, intravenous, intramuscular, intratumoral, rectal, and transdermal.

10. The method of claim 1 wherein the compound is administered to the patient in an amount ranging from about 0.25 μg/ml to about 400 μg/ml, per kilogram of patient weight.

11. The method of claim 1 wherein the compound is administered to the patient in an amount ranging from about 5 μg/ml to about 50 μg/ml, per kilogram of patient weight.

12. The method of claim 6, further comprising the step of administering to the patient another anticancer compound, radiation, or a compound that induces apoptosis.

13. The method of claim 6, further comprising the step of administering herceptin to the patient.

14. The method of claim 6, further comprising the step of administering gossypol or analogs of gossypol to the patient.

15. A method of treatment of cancer, where erbB-2 protein is overexpressed, comprising administering to the subject houttuynum or an analogue thereof selected from a compound from Claims 2-5 in an amount effective to inhibit erbB-2, in combination with a chemodrug selected from doxrubicin, cisplastin, VP-16, and other conventional chemotherapies.

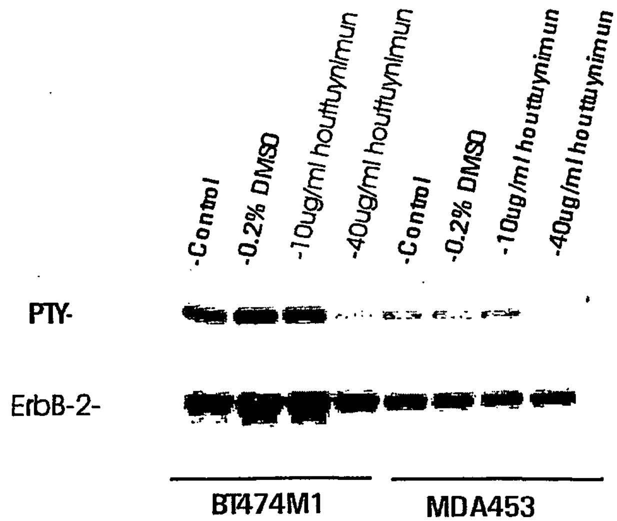


Fig 1. Inhibition of erbB-2 phosphorylation by houttuynimun Bt474m1 and MDA453 cells were treated with different concentrations of houttuynimun for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-PTY or anti-erbB-2.

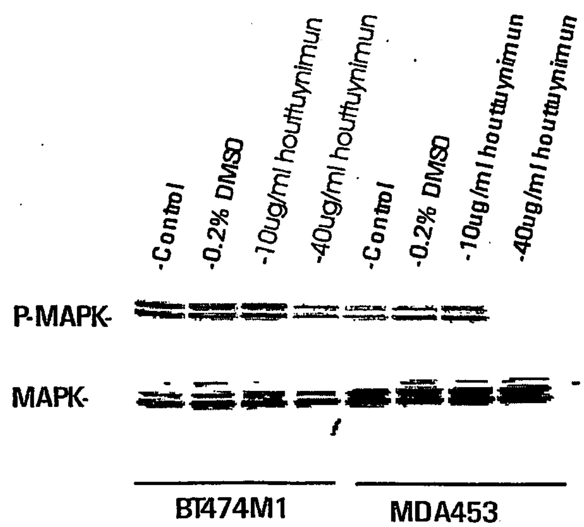


Fig 2. Inhibition of MAPK activation by houttuynimun Bt474m1 and MDA453 cells were treated with different concentrations of houttuynimun for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phospho-MAPK or anti-MAPK antibody.

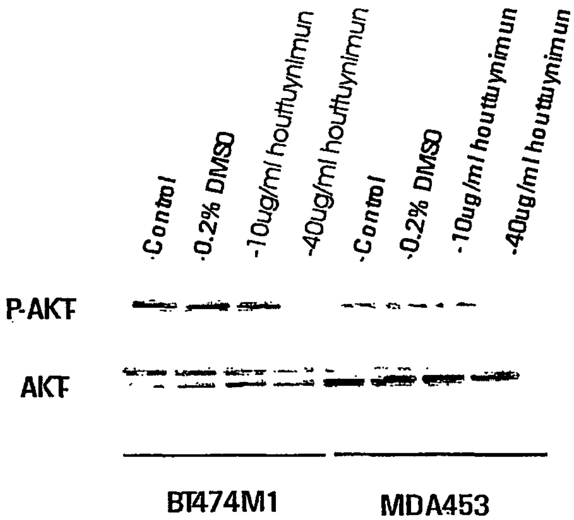


Fig3. Inhibition of akt activation by houttuynimun Bt474m1 and MDA453 cells were treated with different concentrations of houttuynimun for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phospho-akt or anti-akt antibody.

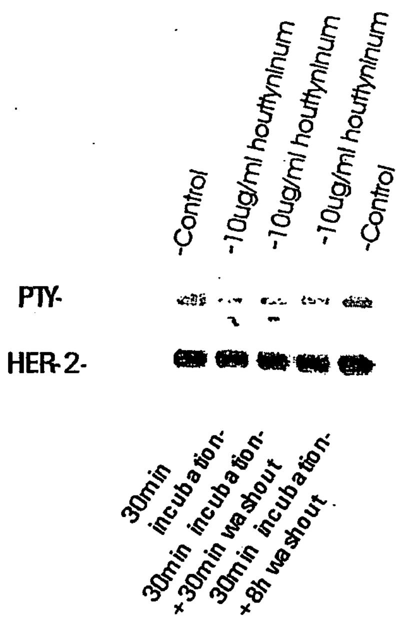


Fig4. Irreversable inhibiton of HER-2 phosphorylation by houttyninum MDA453 cells were treated with 10ug/ml of houttuyninum for 30 min, then was washed with medium and incubated for 30 min or 8h, collect cells and western blot was conducted using anti-PTY or anti-HER-2 antibody.

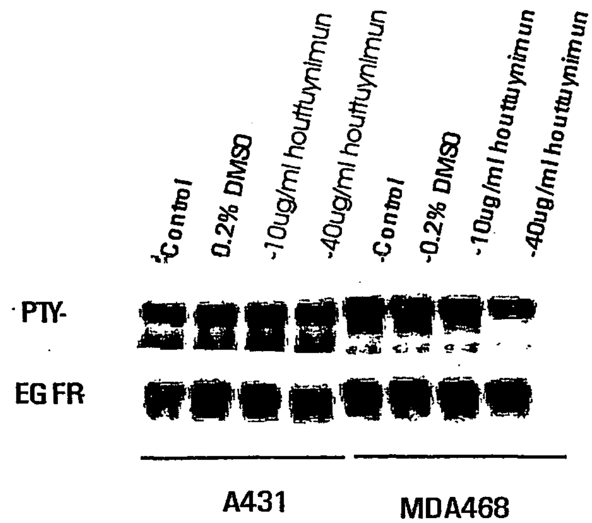


Fig5. Inhibition of EGFR phosphorylation by houttuynimun A431and MDA468 cells were treated with different concentrations of houttuynimun for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phosphotyrosine or anti-EGFR antibody.

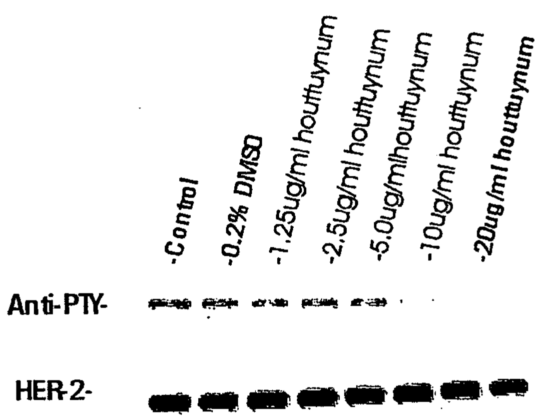


Fig6. Inhibition of HER-2 activation by houttuynum MDA453 cells were treated with different concentrations of houttuynum for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-PTY or anti-HER-2 antibody..

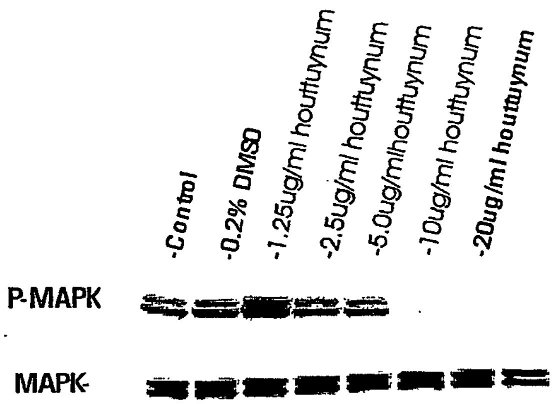


Fig7. Inhibition of MAPK activation by houttuynum MDA453 cells were treated with different concentrations of houttuynum for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phospho-MAPK or anti-MAPK antibody..

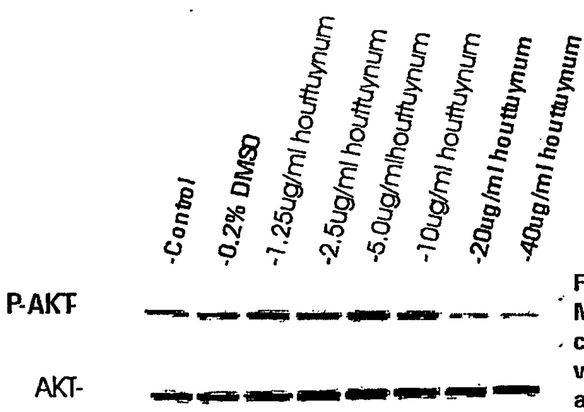


Fig8. Inhibition of AKT activation by houttuynum MDA453 cells were treated with different concentrations of houttuynum for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phospho-AKT or anti-AKT antibody..

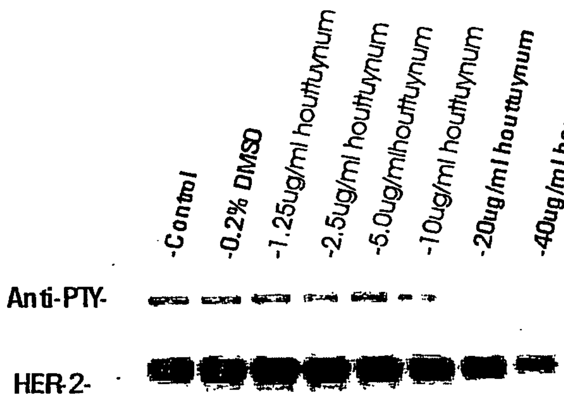


Fig9. Inhibition of HER-2 activation by houttuynum Bt474m1 cells were treated with different concentrations of houttuynum for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-PTY or anti-HER-2 antibody..

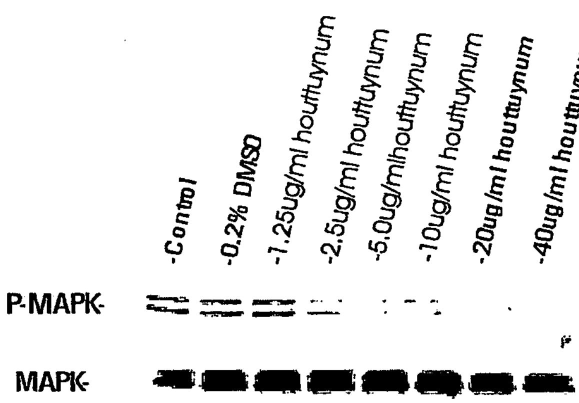


Fig10. Inhibition of MAPK activation by houttuynum Bt474m1 cells were treated with different concentrations of houttuynum for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phospho-MAPK or anti-MAPK antibody..

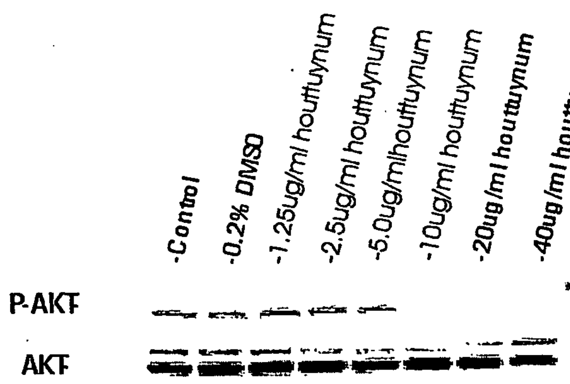
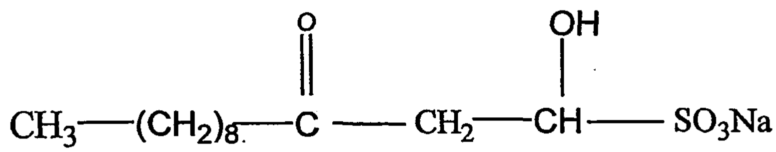


Fig11. Inhibition of AKTactivation by houttuynum Bt474m1 cells were treated with different concentrations of houttuynum for 1h. Cells were collected and lysed. Western blot analysis was conducted and probed with anti-phospho-AKTor anti-AKTantibody..

Figure 12. Structure of Houttounum.

Note, please change the (CH_2)8, 8 into n, whereas n=4-16.



Structure of houttyunum

Figure 13. Effects of the soluble extract houttuynum (YX1, 2mg/ml in original stock) on phosphorylation of erbB-2 in MDA-453 or EGFR in MDA-468 cells.

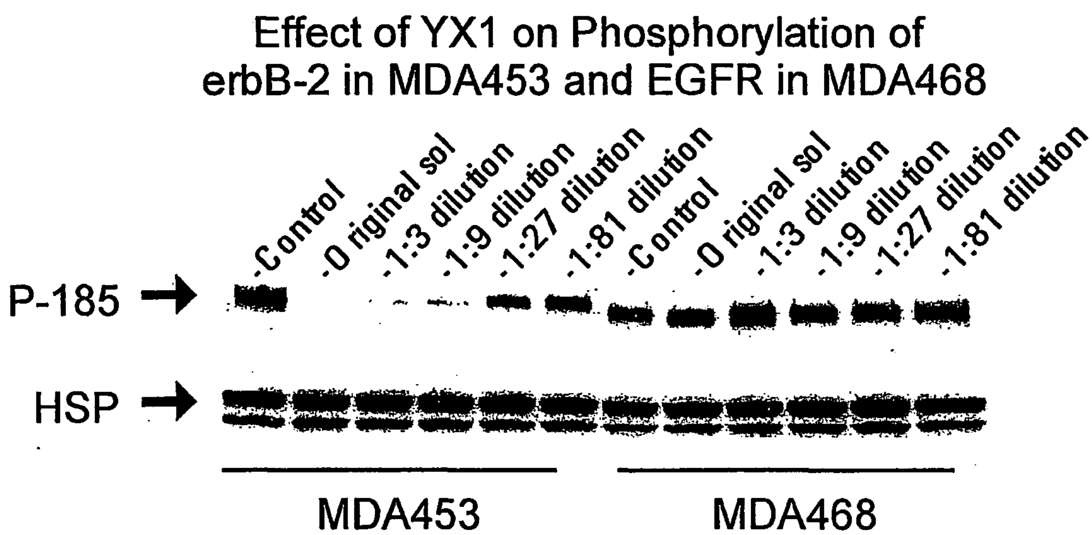
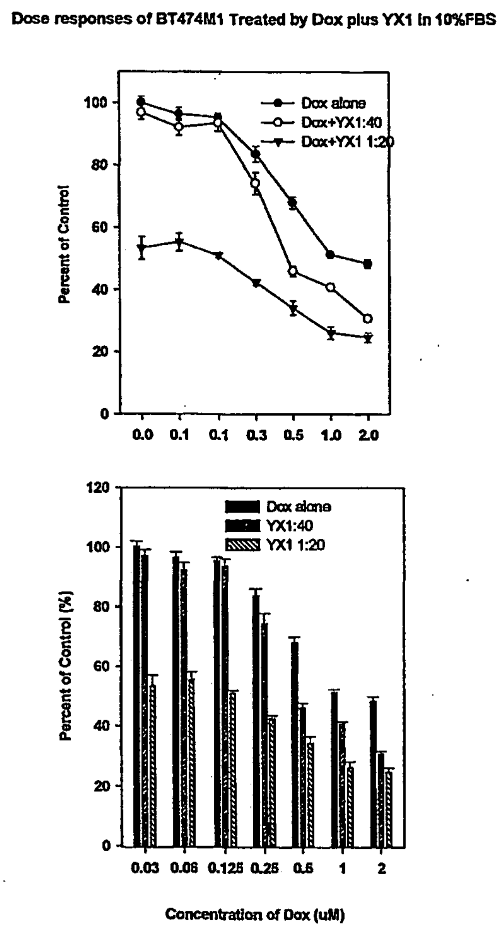


Figure 14. Effects of the soluble extract houttuynum (YX1 2 mg/ml in original stock) on protein expression of erbB-2 in MDA-453 or EGF-R in MDA-468 cells.

Figure 15. Enhanced cytotoxicity of Doxrubicin in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).



Dox+YX1 BT474M1 08 08 2001

Figure 16. Enhanced cytotoxicity of Cisplatin in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).

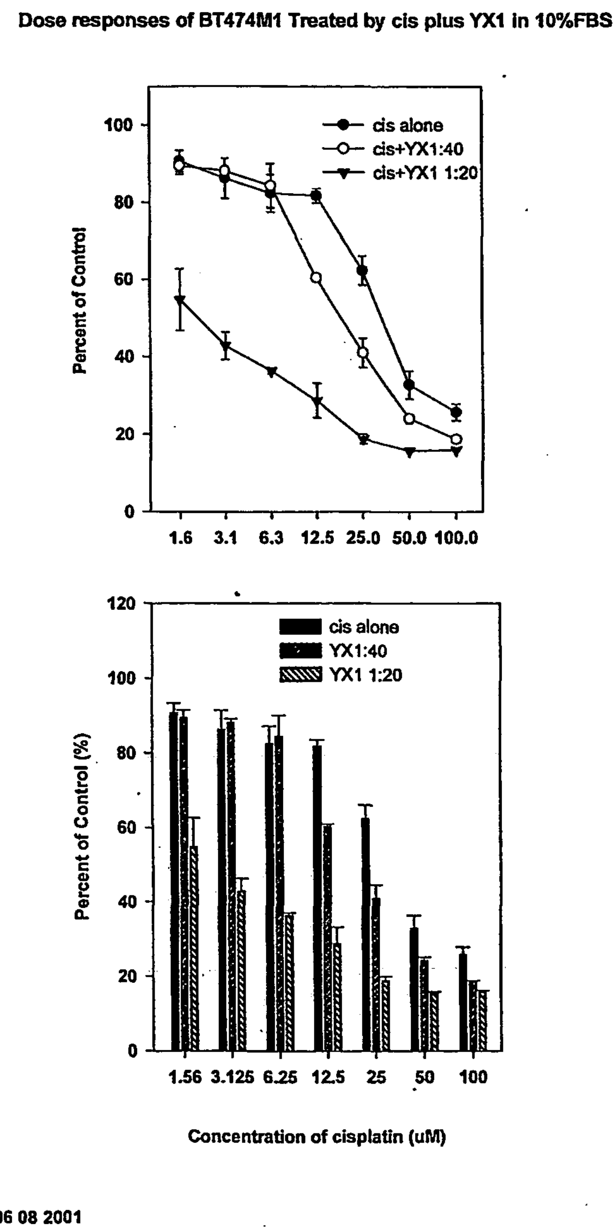
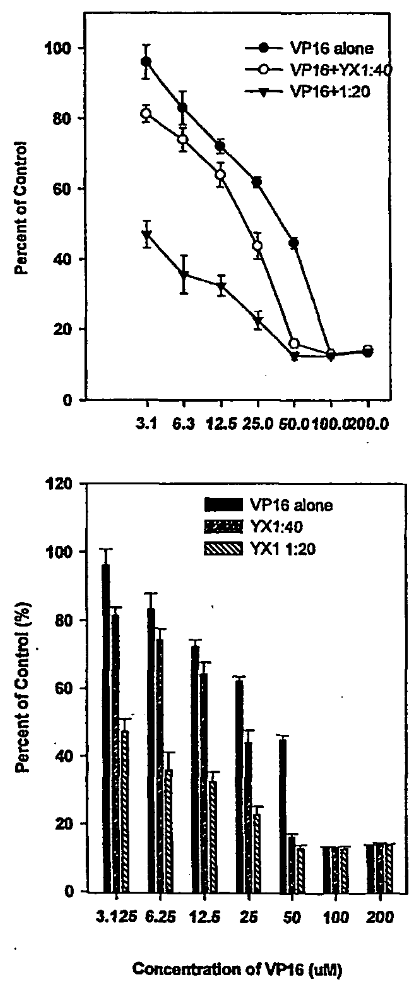


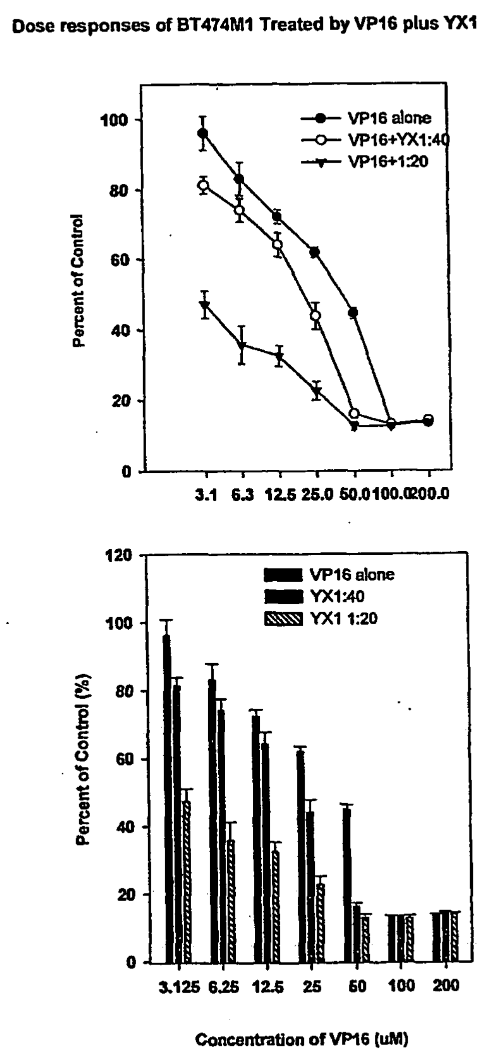
Figure 17. Enhanced cytotoxicity of VP-16 in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).

Dose responses of BT474M1 Treated by VP16 plus YX1 in 10%FBS



VP16+YX1 BT474M1 06 08 2001

Figure 18. Enhanced cytotoxicity of VP-16 in erbB-2 overexpressing human breast cancer cells BT-474 cells by the soluble extract houttuynum (YX1 2 mg/ml in original stock).



VP16+YX1 BT474M1 06 08 2001

Figure 19. Effect of the soluble extract houttuynum (YX1, 2 mg/ml in original stock) or houttuyninum compound (YX2) on *in vivo* tumor proliferation of erbB-2 overexpressing human cancer cell N87.

Effect of YX1and YX2 Treatment on Tumor Growth of Human Cancer Cells N87